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(54) Title: POLYETHYLENEGLYCOL CONJUGATED NANOERYTHROSOMES, METHOD OF MAKING SAME AND USE THEREOF

(57) Abstract

The present invention relates to nanoErythrosomes, a Drug Delivery System (DDS). More specifically, the present invention relates to a new method of production of nanoErythrosomes. Moreover, the present invention relates to nanoErythrosome compositions having a decreased immunogenic potential and to the use thereof in diagnostic and therapeutic methods. The invention further relates to the bioassays using the nanoErythrosome composition of the present invention to diagnose or prognose a predetermined condition in an animal, as well as kits containing those nanoErythrosomes compositions.

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TITLE OF THE INVENTION

POLYETHYLENEGLYCOL CONJUGATED NANOERYTHROSOMES, METHOD OF MAKING SAME AND USE THEREOF

FIELD OF THE INVENTION

The present invention relates to nanoErythrosomes, a Drug Delivery System (DDS). More specifically, the present invention relates a new method of production of nanoErythrosomes. Moreover, the present invention relates to nanoErythrosome compositions having a decreased immunogenic potential and to the use thereof in diagnostic and therapeutic methods. The invention further relates to the bioassays using the nanoErythrosome compositions of the present invention to diagnose or prognose a predetermined condition in mammal, as well as to kits containing these nanoErythrosome compositions of the present invention.

BACKGROUND OF THE INVENTION

NanoErythrosomes (nEryt) are small vesicles that are produced from erythrocytes (red blood cells) which are treated in a low-salt solution to remove their haemoglobin content. Subsequently, these haemoglobin-free erythrocytes (ghosts) are extruded (intruding filtration under vacuum) to form small vesicles having a mean diameter of about 100 nm (USP 5,653,999). The nanoErythrosome can in essence be considered lipoproteosomes (vesicles constituted of both lipids and proteins) by analogy with liposomes. They nevertheless represent totally distinct scientific universes when compared to the almost exclusively

lipid-based liposomes. The nanoErythrosomes are buoyant vesicles which remain in suspension for prolonged periods of time because of their high surface to volume ratio (approximately 80-fold higher than the parent red blood cell). The nanoErythrosome can carry various types of molecules such as drugs or peptides that will be transported through the bloodstream to various targets. They can be coupled to antibodies or peptide ligands for selective delivery to cells, for example. Further, the nanoErythrosomes can encapsulate a biologically relevant substance and/or alternatively a biologically relevant molecule can be coupled thereto. NanoErythrosomes are thus very versatile bioactive drug carriers or drug delivery systems (DDS).

NanoErythrosomes have been shown to have advantages over other DDS and could constitute a breakthrough in biopharmacology. For example, numerous drugs must be given repeatedly to maintain their blood concentration at an optimal therapeutic level, for extended periods of time. To do so, several methodologies are currently being tested, such as the use of albumin micro spheres, acrylic micro spheres, liposomes, magnetic polymers, lectins and mAbs. Most of those technologies are still in development and their applications are more or less limited to a group of specific products such as antineoplastic drugs and/or to a targeted organ such as the liver or spleen. Although the results obtained with some of these drug carriers are encouraging, the substances composing those carriers are often recognized as foreign materials or haptens by the immune system and consequently, they eventually trigger deleterious immunological reactions.

Moreover, some of these carriers are rapidly captured and destroyed either by the liver or the spleen. The drugs are then

liberated in high concentration directly in those organs, often resulting in toxic effects.

Advantages of nanoErythrosomes comprise: 1) they significantly modify the metabolism of the drug they transport by preventing their elimination via the liver or kidney; 2) they can encapsulate or bind numerous biologically relevant molecules or bioactive substances; 3) they can be used as a progressive drug-releasing system for several types of molecules, such as antineoplastics, phototherapeutic agents and peptides, thus maintaining an optimal concentration of the drug in the bloodstream for extended period of time; and 4) they can reduce the need for high dose instantaneous administration of large quantities of drugs into the bloodstream, thereby reducing deleterious effects such as nausea and vomiting which are caused by high blood concentrations of toxic drugs (USP 5,653,999).

However, the nanoErythrosomes, albeit to a reduced level as compared to other carriers can still, elicit on immune response in a mammal. Moreover, they are destroyed by gastric juices and hence could not be efficiently used in an unmodified from for per os administration.

NanoErythrosomes, by nature, are constituted of both lipids and proteins. The presence of proteins and lipids such as phosphatidylserine in the nEryt could lead, if used in non-autologous applications (a mammal is receiving nanoErythrosomes from another non compatible mammal, as opposed to autologous administration, whereby the blood donor and recipient are the same or at least the donor and recipient are compatible) to immunological reaction of the recipient mammal. Those undesirable protein-protein interactions could seriously

limit the potential for therapeutic, diagnostic and commercial applications of technologies based on nanoErythrosomes. In therapeutic applications, for example, nanoErythrosomes could lead to deleterious immune responses. Moreover, if it is used for diagnostic applications they could be responsible for high background. There thus remain a need to find strategies that will abrogate those undesirable reactions. Furthermore, there remains a need to provide a nEryt composition which is more stable at low pH conditions (such as those of the stomach).

The use of Polyethyleneglycol (PEG) to reduce the immunogenicity of proteins as been previously reported and is well known in the art (USP 5,595,732). The use of PEG to coat liposomes has also been reported (USP 5,593,622 and USP 5,620,689). However, it remains to be determined whether nanoErythrosomes could withstand PEG coupling without collapsing or whether they could retain their biological activity. There thus remain a need to provide a nanoErythrosome composition which possesses a reduced immunological potential, yet retains its biological activity as a drug delivery system.

The coupling of an antibody to a drug delivery system in order to specifically target the DDS to a specific site recognized by the antibody has been reported. For example, USP 5,620,689 teaches CD-19 antibody-coupled liposomes. Whether coupling of antibodies to nEryt maintains the integrity of nEryt as yet to be determined.

There thus remains a need to provide nEryt compositions which are specifically targeted to cells or tissue by way of a ligand conjugated thereto.

The present invention seeks to meet these and other needs.

The present description refers to a number of documents, the content of which is herein incorporated by reference.

SUMMARY OF THE INVENTION

This invention concerns nanoErythrosomes as a diagnostic or therapeutic tool. More specifically, the present invention relates to nanoErythrosomes which have been coupled to a ligand, such as an antibody or portion thereof, in order to enable a targeting of the nanoErythrosome to a specific receptor for this ligand, such as an antigen (recognized by an antibody). By further coupling a biologically relevant substance to such a nanoErythrosome, or by encapsulating the biologically relevant substance in the ligand coupled nanoErythrosome, a targeting of the biologically relevant substance to the receptor which is recognized by the ligand coupled to the nanoErythrosome is now possible. In one embodiment, the invention relates to a nEryt composition which comprises a nanoErythrosome coupled to an antibody which recognizes the cancer antigen gp54/TROP-2, the antibody-coupled-nanoErythrosome encapsulating a bioactive agent. In a particular embodiment, the antibody coupled antibody encapsulates a fluorochrome. In a particular preferred embodiment, the antibody coupled nEryt encapsulates an anti-cancer molecule.

The invention also relates to a method of preparing such ligand-coupled nanoErythrosomes and/or ligand-coupled nanoErythrosomes encapsulating a biologically relevant substance.

In a particular embodiment, the invention relates to a method of preparing antibody-coupled-nEryt compositions encapsulating or not a biologically relevant or bioactive substance.

The invention further relates to a complex comprising a nanoErythrosome coupled to an antibody or portion thereof, wherein the antibody or portions thereof targets the nanoErythrosome to an antigen recognized by the antibody or portion thereof.

Additionally, the invention relates to a method for encapsulating a biologically relevant substance into a nanoErythrosome comprising a cycle of heat shock. In one embodiment, the encapsulation or capture method comprises cycles of freeze thawing on ice, dry ice or other freezing mixture (for example, ice-sodium chloride (-20°C approx.), acetone-dry ice (-78°C), preferably in liquid nitrogen.

The present invention also relates to a nanoErythrosome composition having a significantly reduced immunogenic potential. More particularly, the invention relates to nEryt-PEG compositions.

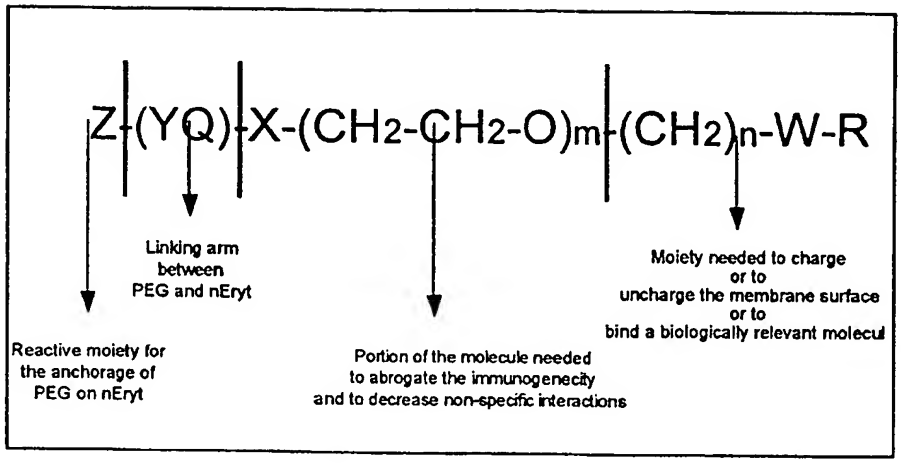
Further the present invention relates to a nEryt composition which can be administered per os. More particularly, the invention relates to a PEG-nEryt composition which can be administered per os.

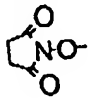
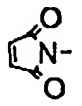
Wherein the preferred embodiment of this invention is demonstrated with a specific antibody, and with specific biologically relevant substances, the invention is not so limited, since a person of ordinary skill will recognize that the method of the present invention can be adapted to the coupling of numerous types of ligands including antibodies or portions thereof. The term "ligand" should thus be

interpreted in a broad sense as defining a substance having high affinity for a receptor. Numerous types of ligands are well known to the person of ordinary skill. Similarly, a multitude of different biological relevant substances can be encapsulated in the nanoErythrosome in accordance with the present invention, and/or coupled to the nanoErythrosomes in accordance with the present invention.

Surprisingly, it has been found that the conjugation of polyethyleneglycols derivatives could be useful towards reducing the immunogenic potential of nEryt without abrogating the biological activity of the nEryt vesicle. Although as previously mentioned, a number of publications and patents exist on the subject of PEG coupling to proteins or liposomes, the applicants are the first to show that nanoErythrosomes can be coupled to PEG without compromising significantly the integrity of the nEryt vesicles. It must be pointed out that the nEryt-PEG coupling technology was difficult to develop, because of the number of parameters involved.

To help set a framework as to the complexity of the endeavor of nEryt-PEG coupling, the following general formula of PEG and its interpretation is presented.



"Z" is the group that will be responsible for the conjugation of PEGs to the proteins present in the nEryt membrane. The composition of that reactive group is very important for the control of the kinetic of PEGs conjugation to available reactive groups, such as amino groups, such as those of lysine residues. Two types of reactive groups targeting two families of nucleophiles present on proteins were used for that purpose i) NH_2 groups (i.e lysine residues), exemplified by activated esters such as succinimide () esters; and ii) SH groups (i.e cysteine residues or iminothiolane derivative of lysine), exemplified by maleimides (), 2-thiopyridyl () derivatives and

disulfide groups. It shall that the nEryt membrane can be modified according to known methods by the person of ordinary skill to provide and/or modify reactive groups which can serve as partners conjugating PEG or other substance.

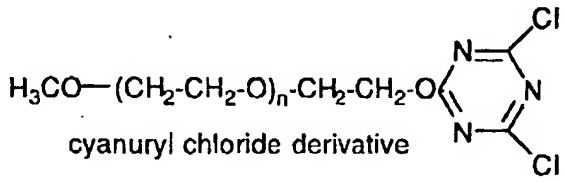
In a preferred embodiment, Z is a member selected from the group consisting, if YQ= CH=CH or -C(R")=CH_2, of COOH, HO (aldehyde), H, OH (hydroxyl), NH_2, SH. If Y= -C=(O)-, Z= H, N_3, OH, CH_3, -NH-NH_2, an anhydride, a mixed anhydride or an «activated ester» such as known by the man of art and defined and exemplified by M. Bodansky in Principles of peptides synthesis; chapter II, Activation and coupling (Haftner et al., 1984, Eds. Springer-Verlag, New York, pp 9-52).

Some examples of activating groups useful in binding PEG to nEryt include:

cyanogen bromide (BrCN) , des amino acid esters (Zalipsky et al., 1984, J. Macromol. Sci. Chem. A21: 8339; Mutter et al., 1979, The Peptides; Gross et al., J., eds., 2, p. 285, Academic Press, New York), hydrazine derivates, Rubinstein, 1978, U.S. Patent 4,101,380; Davis et al., 1979, U.S. Patent 4,179,337 and Persson et al., 1988, J. Chromatog. 457: 183; succinimidyl carbonate derivates, Miron et al., 1993, Bioconjugate Chem. 4: 568; Zalipsky et al., 1993, Bioconjugate Chem. 4: 296 and Zalipsky et al., 1991, Polymeric Drugs and Drug delivery Systems; Dunn et al., eds) ACS, Washington, DC); oxycarbonylimiidazole derivates, Allen et al., 1991, Biochem. Biophys. Acta 1066: 29; and Tondelli et al., 1985, J. Controlled Release 1: 25); nitrophenyl carbonate derivates, Satore et al., 1991, Appl. Biochem. Biotech. 27: 45; tresylate derivates, Klibanov et al., 1991, Biochem. Biophys. Acta 1062:142, Delgado et al., 1990, Biotech. Appl. Biochem. 12:119); maleimide derivates, Kogan, 1992, Synthetic

Commun. 22:2417 and Romani et al., 1984, Chemistry of Peptides and Proteins, Volter et al., eds, 2, p 29, Walter de Gruyter, Berlin).

The "YQ" portion of the molecule is a linking arm allowing the conjugation of PEG to proteins. The groups used as YQ are preferably composed of $(CH_2)_n$, where n = 1 to 8 carbon atoms, preferably 2 to 5 carbon atoms. The use of a particular n can be readily adapted by the skilled artisan, depending on the particular nEryt-PEG composition desired and use thereof. In general, when n is more than 8, the molecule tends to be too liposoluble. The YQ group is a critical group since there are very few ways to directly conjugate the OH group of polyethyleneglycols to proteins. One exception, is cyanuryl chloride derivatives that has been used in preliminary experiments.



cyanuryl chloride derivative

In a preferred embodiment, YQ is a member selected from the group consisting of: CH=CH, $-C(R'')=CH_2$ (where R'= lower alkyl of 1 to 5 carbon atoms), cyanuric chloride, cyanogen halides (Br or Cl), and other OH« activating agents» such as mesyl, tosyl groups. If Y is a member selected from the group of lower alkyl $[(CH_2)_n$; n = 1-7]; then Q= -C=(O), N, S

"X" atom can be an oxygen (ether), a sulfur (thioether) atom or a -O-C(O) (ester), a -N-C(O) (amide) and a -S-C(O) (thioester). Thioalkoxy, alkoxy bonds and amides are biologically stable, however, ester and thioester linkages are much more labile since they are

hydrolyzed in biological media with a kinetic that is more or less rapid. The hydrolytic kinetic is related to the lenght of the YQ portion. However, they may prove very useful in the design of nEryt used as a slow releasing device. It will be determined by the person of ordinary skill whether a stable bond or more labile bond between the nEryt and the biologically relevant molecule or bioactive agent. Although in general a stable bond is preferable, a non-limiting utility for a more labile bond, includes a situation in which after having targeted the nEryt composition to a specific location, phagocytosis of the nEryt rendered immunogenic by way of breakage of the bond linking same to the PEG moiety and possibly encapsulating bioactive agent by macrophages is desirable.

The $(CH_2\text{-}CH_2\text{-}O)_m$ moiety of the molecule is the polyethyleneglycol itself. m could be anything between 1 and 500. Thus, PEGs having molecular weights from 350 to 10,000 can be used, preferably PEGs of between 1,000-10,000, more preferably 2,000-5,000 molecular weight.

Finally, the $[(CH_2)_n\text{-}W\text{-}R]$ group faces the media surrounding the nEryt. The nature of this group evidently critical for the present applications. For example, $(CH_2)_n$-W-R should preferably be an inert group such as OCH_3 (WR) in order to abrogate of the immune responses, in applications where nEryt is involved as a simple carrier (DNA vaccines) or for absorption through mucosas (lung, intestine). However, for diagnostic applications requiring a specific deplacement of nEryt in an electric fields (microchips technologies), $(CH_2)_n$-W-R must be a electropositively (i.e.-NH_2) or electronegatively (i.e.-COOH) charged group. $(CH_2)_n$-W-R could also be a group such as SH, 2-thiopyridyl or maleimide when the polyethyleneglycol derivative is required to bind a

biologically relevant molecule such as antibodies to target a cell or a specific organ while abrogating the intrinsic immunogenicity of the nEryt. In view of the above, it will be readily clear to the person of ordinary skill that the nature of the [$(CH_2)_n$-W-R] group will be adapted to meet the particular needs of a specific application or use. Preferably, n= 1 to 7 carbon atoms.

W is a member selected from the group consisting of: O, N, S, -C(=O).

If W= -O-, R is a member selected from the group consisting of: H, lower alkyl or cycloalkyl of 1 to 7 carbon atoms, -C(=O)-R, where R is a polyamine derivative such as spermine, spermidine or putresceine

If W= -N-, R is a member selected from the group consisting of: H, lower alkyl or cycloalkyl of 1 to 7 carbon atoms, -C(=O)- -C(=O)-R, where R is a polyamine deriving from spermine, spermidine or putresceine or a lower alkyl chain of 1 to 6 carbon atoms bearing one or two COOH, SO_3H or PO_4 groups

If W= -S-, R is a member selected from the group consisting of: H, lower alkyl or cycloalkyl of 1 to 7 carbon atoms, -C(=O)- -C(=O)-R, where R is a polyamine deriving from spermine, spermidine or putresceine.

If W= -C(=O)-, R is a member selected from the group consisting of: H, lower alkyl or cycloalkyl of 1 to 7 carbon atoms, -O- -C(=O)-R, where R is a polyamine deriving from spermine, spermidine or putresceine.

WR is a member selected from the group consisting of: COOH, PO_4, SO_3H.

It will be clear from the above that the choice of PEGs that can be used in the context of nEryt modifications is complex. Moreover, when the modified PEGs are reacted to nEryt, other parameters of utmost importance must be considered, an example of which includes the percentage of PEG substitution in relation to the number of available reactive groups (i.e NH_2 and/or SH groups) present on the membrane. If the substitution is too high, the nEryt can collapse on itself or become useless for further tasks such as capture or conjugation of bioactive agent in or to by the nEryt. Conversely, if the substitution is too low, the nEryt can remain too immunogenic (which can be determined detrimental in therapeutic applications) or unprotected against various types of undesired interactions leading to non-specificity of the carrier for the target (undesirable in diagnostic interactions). The percentage of reactive groups on the nEryt membrane (i.e. SH or NH_2) which is substituted by PEG can be readily adapted by the person of ordinary skill now cognizant of the present invention. Preferably, 2-30% of the reactive groups of the membrane of the nEryt will be substituted with PEG, more preferably 2-15%.

nEryt can be prepared from blood from various mammalian sources (mouse, rat, rabbit, ovine, bovine, echine, porcine, human, and the like). The blood is collected on anticoagulant (heparine, EDTA, citrate, and the like). In accordance with one embodiment of the present invention, the blood is centrifugated and the plasma and the buffy coat are discarded. The packed erythrocytes are resuspended in phosphate buffer (PBS; 150 mM NaCl, 5.0 mM K_2HPO_4/KH_2PO_4, pH 7.4) to their initial volume of blood. The erythrocytes are washed four times and resuspended at the concentration of 2×10^9 cells/ml.

The erythrocytes in suspension can be depleted of their hemoglobin as previously described in UPS 5,653,999. However, the present invention provides a more efficient, more quantitative, simpler and more easily scalable method of ghost preparation. In accordance therewith, the erythrocytes in suspension are depleted of their hemoglobin by size-exclusion chromatography on a column containing the appropriate chromatographic gel Sepharose CL6B or Sephacryl S 400 high resolution using an hypotonic (2 to 5 mM) aqueous buffered solution at pH 8 to 11 without di and/or trivalent cations, a significant modification with respect to the previous protocol of Wood (1987, Methods in Enzymol. 149:271-280; and of USP 5,653,999). The recovery of the white ghosts from red blood cells is almost quantitative.

The next step of the production is providing a significant modification in the method of production of nanoErythrosomes as compared to that of USP 5,653,999. The pH of the hypotonic suspension of white ghosts is brought to 7.4 is important to point out the essential role of hypotonic conditions and the absence of cations in the method of the present invention. Failure to comply with these requirement will totally compromise the production of nEryt. The suspension is filtered through a filter of about 0.45μm immediately followed by a filtration through a filter of about 0.22μm. Notably, in the method of the present invention, no extrusion is used. Furthermore, no multiple extrusions through a 1μm filter are required. Indeed, instead of applying pressure on the suspension to push the ghosts through the filter, the suspension is filtered under vacuum through two filters (i.e teflon) of about 0.45 and 0.22 μm, respectively. The yield of recovery of nEryt is also almost

quantitative. As a maximum of 10% of nEryt are lost , preferably less than 5%. Using the method of USP 5,653,999, 20% or more of nEryt are lost. All manipulations are preferably performed under sterile conditions.

The diluted suspension ((10^{12} nEryt per ml) can be concentrated by several means commonly known by the person of ordinary skill i) either centrifugation of the aqueous solutions or on trehalose, sucrose gradients (i.e. 20,000g X 20 min.); ii) dialysis in cellulose acetate tubing having cut-off between 200,000 and 1X 10^6 molecular weight; iii) concentration using an Amicon system; iv) lyophilization using trehalose, sucrose or raffinose solutions (ratio mg of sugar of nEryt [proteins] is between 1 to 100:1). These technologies are applicable to unmodified nEryt, nEryt having a bioactive agent entrapped therein, nEryt having a bioactive agent covalently bound thereto and nEryt having a bioactive agent entrapped therein and having a bioactive agent covalently bound thereto.

As used herein, the terms "encapsulated", "entrapped" and "capture" when relating to a bioactive agent are defined broadly as it is extremely difficult to assess where bioactive agent is specifically located following the "treatment". Indeed, it is difficult to assess whether the bioactive agent is inside the nEryt or deeply bonded to the membrane. Nevertheless, since experiments (i.e. with DNA) demonstrate that the DNA is protected from degradation (see below), it will be assumed that the bioactive agents are entrapped/captured by the nEryt.

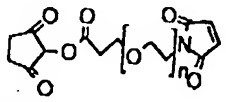
The entrapment/capture procedure is simple: nEryt are suspended in an hypotonic or isotonic solution, cation-free solution (di and/or trivalent) containing the highest concentration possible of the bioactive agent to be entrapped. The capture of the molecule can take

place at a low rate at room temperature. Preferably, it will be carried out more efficiently and quickly by quick freezing the nEryt-bioactive agent solution and allowed to warm up at rom temperature. The nEryt-bioactive agent solution on the frozen ice, dry ice or other freezing mixture which are well known to the person of ordinary skill. Non-limiting examples of freezing mixtures include ice-sodium chloride (approximately -20°C), acetone-dry ice (approx. -78°C) and the like. Preferably, the freezing mixture is liquid nitrogen. The free bioactive agent can then be separated from captured ones through various processes adapted to their chemical nature (centrifugation-wash cycles, exclusion chromatography, dialysis, and the like) and adaptable by the person of ordinary skill. nEryt having captured molecules can be concentrated (lyophilized) or used in latter processes such as conjugation of PEGs, ligands, antibodies to their membrane surface. It is of utmost importance to avoid the presence of di and/or trivalent cations such as Mg, Mn, Zn, Ca, and the like in the suspension. The presence of such cations seems to seal the membrane of nEryt such that the capture of bioactive agents is decreased in most cases. It will be recognized by the person of ordinary skill that this "sealing" property of the di and/or trivalent cations on the nEryt membrane can be used advantageously in certain conditions to further diminish the leakage of a bioactive agent capture in the nEryt. Such types of sealed nEryt having captured nucleic acid sequences are also within the scope of the present invention.

Various biologically important molecules such as ligands including antibodies, and polyethyleneglycols or chemical modifiers such as iminothiolane can now be conjugated to the membrane surface of nEryt. In a preferred embodiment of diagnostic applications monoclonal

antibodies such as 48-127, and M344 can be conjugated to nEryt. The antibodies can be conjugated as described above using -NH_2 groups present on lysine residues, or -SH groups of cysteine residues on the nEryt membrane. The conjugation can take place through numerous techniques using heterobifunctional linking arms such as, for example,

SMCC ( ) or an heterobifunctional PEGs of the following general



structure ( ), wherein n = 2 to 100.

As alluded to above the chemistry of nEryt permits a modification of the proteins present in the membrane, for example, i) iminothiolane can add SH groups to the membrane. That reaction allows the conjugation of PEG or antibodies using electrophilic groups such as maleimides (ex: SMCC and the heterobifunctional PEGs), ii) addition, for example, of anhydrides such as succinic, cis aconitic, citraconic to nEryt will add -COOH groups that introduce negative charges on the surface. This can be useful for diagnostic applications requiring the use of electric fields to perform the separation of unbound from bound nEryt. iii) addition, for example, of polyamines derives such as spermine, putrescine, spermidine or polylysine derivatives to nEryt will add -NH_2 groups that introduce positive charges on the surface that will be useful for diagnostic applications requiring the use of electric fields to perform the separation of unbound from bond nEryt. Such a chemistry can also

be possible by conjugating PEGs having a R_2 group where R_2= -COOH, PO_4^{2-}, SO_3H, NH_2 or substituted with polyanionic or polycationic molecules such as spermine, heparine, etc (see general formula of PEG).

In general, the polyethyleneglycol derivatives of the present invention can be represented by the general formula:

$$Z\text{-}(YQ)\text{-}X\text{-}(CH_2\text{-}CH_2\text{-}O)_m\text{-}(CH2)_n\text{-}W\text{-}R$$

wherein

YQ is a member selected from the group consisting of: CH=CH, -C(R")=CH_2 (where R'= lower alkyl of 1 to 5 carbon atoms), cyanuric chloride, cyanogen halides (Br or Cl), and other OH« activating agents» such as mesyl, tosyl groups. If Y is a member selected from the group of lower alkyl [$(CH_2)_n$; n = 1-7]; then Q= -C=(O), N, S

Z is a member selected from the group consisting, if YQ= CH=CH or -C(R")=CH_2, of COOH, HO (aldehyde), H, OH (hydroxyl), NH_2, SH. If Y= -C=(O)-, Z= H, N_3, OH, CH_3, -NH-NH_2, an anhydride, a mixed anhydride or an «activated ester» such as known by the man of art and defined and exemplified by M. Bodansky in Principles of peptides synthesis; chapter II, Activation and coupling (Haftner et al., 1984, Eds. Springer-Verlag, New York, pp 9-52).

Some examples of activating groups useful in binding PEG to nEryt include:

cyanogen bromide (BrCN) , des amino acid esters (Zalipsky et al., 1984, J. Macromol. Sci. Chem. A21: 8339; Mutter et al., 1979, The Peptides; Gross et al., J., eds., 2, p. 285, Academic Press, New York), hydrazine derivates, Rubinstein, 1978, U.S. Patent 4,101,380; Davis et al., 1979, U.S. Patent 4,179,337 and Persson et al., 1988, J. Chromatog. 457: 183;

succinimidyl carbonate derivates, Miron et al., 1993, Bioconjugate Chem. 4: 568; Zalipsky et al., 1993, Bioconjugate Chem. 4: 296 and Zalipsky et al., 1991, Polymeric Drugs and Drug delivery Systems; Dunn et al., eds) ACS, Washington, DC); oxycarbonylimiidazole derivates, Allen et al., 1991, Biochem. Biophys. Acta 1066: 29; and Tondelli et al., 1985, J. Controlled Release 1: 25); nitrophenyl carbonate derivates, Satore et al., 1991, Appl. Biochem. Biotech. 27: 45; tresylate derivates, Klibanov et al., 1991, Biochem. Biophys. Acta 1062:142, Delgado et al., 1990, Biotech. Appl. Biochem. 12:119); maleimide derivates, Kogan, 1992, Synthetic Commun. 22:2417 and Romani et al., 1984, Chemistry of Peptides and Proteins, Volter et al., eds, 2, p 29, Walter de Gruyter, Berlin).

$O(CH_2\text{-}CH_2O)_m$ is a polyethyleneglycol derivative where m= 2 to 500.

n= 1 to 7 carbon atoms.

W is a member selected from the group consisting of: O, N, S, -C(=O).

If W= -O-, R is a member selected from the group consisting of: H, lower alkyl or cycloalkyl of 1 to 7 carbon atoms, -C(=O)-R, where R is a polyamine derivative such as spermine, spermidine or putresceine

If W= -N-, R is a member selected from the group consisting of: H, lower alkyl or cycloalkyl of 1 to 7 carbon atoms, -C(=O)- -C(=O)-R, where R is a polyamine deriving from spermine, spermidine or putresceine or a lower alkyl chain of 1 to 6 carbon atoms bearing one or two COOH, SO_3H or PO_4 groups

If W= -S-, R is a member selected from the group consisting of: H, lower alkyl or cycloalkyl of 1 to 7 carbon atoms, -C(=O)-

-C(=O)-R, where R is a polyamine deriving from spermine, spermidine or putresceine

If W= -C(=O)-, R is a member selected from the group consisting of: H, lower alkyl or cycloalkyl of 1 to 7 carbon atoms, -O--C(=O)-R, where R is a polyamine deriving from spermine, spermidine or putresceine.

WR is a member selected from the group consisting of: COOH, PO_4, SO_3H.

Ya in the general formula presented above can be a number or reactive agents such as cyanuric chloride derivatives, aldehyde, succinide, benzinidazole, symetric disulfide, heterobidunctional PEG and the

From the instant disclosure, it shall be understood that the designations "biologically relevant substance" and "bioactive agent" are used in a broad sense purposely so as to comprise, without being limited thereto, drugs, molecules, peptides, proteins, nucleic acid sequences, and fluorophores or other labelling molecules, and PEGs. It shall be understood that the term "PEG" is intended to include PEG derivatives.

The nEryt of the invention may be coupled to bioactive agents to form carriers for such agents. In particular the vesicles may be coupled to bioactive agents such as drugs, to provide a carrier for administration of the bioactive agent so that the bioactive agent may be efficiently delivered to the location in the body where the bioactive agent is required. The vesicles are natural materials, biodegradable, non-immunogenic or having moderate immunogenic potential, non-toxic and non-pyrogenic, fully compatible with blood, and adapted for autologous

administration. In the specification and appended claims, the term autologous administration should be interpreted as meaning that the nanoErythrosomes administered to a mammal have been prepared from red blood cells obtained from compatible red blood cells or blood supply (including an administration to and from the same mammal; i.e. the same patient). Although it is preferable to reduce the immunogenic potential of the nEryt when contemplating a non-autologous administration of nanoErythrosomes, an administration of nEryt without treatment to reduce their immunoreactivity, to immunosuppressed or non-immunosuppressed mammals is also contemplated in certain situations.

The coupling is achieved with a coupling agent having a first group reactive with a reactive site of the vesicle and a second group reactive with a reactive group on the bioactive agent. Numerous methods of coupling a bioactive agent to the nanoErythrosome exist and are well known in the art. These include, but are not limited thereto, to the use of well known crosslinking reagents, such as bifunctional reagents of homobifunctional or heterobifunctional type:

Azidobenzoyl Hydrazide, *N*-5-Azido-2-nitrobenzoyloxysuccinimide *N*-[4-(*p*-Azidosalicylamido)butyl]-3'-[2'-pyridyldithio]propionamide, *p*-Azidophenyl glyoxal monohydrate, 4-[*p*-Azidosalicylamido]butylamine 1-[*p*-Azidosalicylamido]-4-[iodoacetamido]butane, Bis-[ß-(4-Azidosalicylamido)ethyl]disulfide, Bismaleimidohexane, Bis[sulfosuccinimidyl] suberate, Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone, Bis[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone, *N*-γ-

maleimidobutyryloxy-succcinimide ester, *N*-γ-maleimidobutyryloxy-sulfosuccinimide ester *N*-Hydroxysuccinimidyl-4-azidobenzoate, *N*-Hydroxysulfosuccinimidyl-4-azidobenzoate, *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester *m*-Maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester, 4-[*N*-Maleimidomethyl]-cyclohexane-1-carboxylhydrazide•HCl, 4-(4-*N*-Maleimidophenyl)-butyric acid hydrazide•HCl, *N*-Hydroxysuccinimidyl-4-azidosalicylic acid, *N*-Hydroxysulfosuccinimidyl-4-azidosalicylic acid Sulfosuccinimidyl[4-azidosalicylamido]hexanoate, 3-[2-Pyridyldithio]propionyl hydrazide, *p*-Nitrophenyl-2-diazo-3,3,3,-trifluoropropionate, 2-Diazo-3,3,3,-trifluoropropionylchloride, *N*-succinimidyl[4-azidophenyl]1,3'-dithiopropionate, Sulfosuccinimidyl[4-azidophenyldithio]propionate, Sulfosuccinimidyl 2-[7-azido-4-methycoumarin-3-acetamide]ethyl-1,3'-dithiopropionate, Sulfosuccinimidyl-2-[*m*-azido-*o*-nitrobenzamido]ethyl-1,3'-dithiopropionate, *N*-Succinimidyl-6-[4'-azido-2'-nitrophenylamino]hexanoate, Sulfosuccinimidyl-6-[4'-azido-2'-nitrophenylamino]hexanoate, Sulfosuccinimidyl-2-[*p*-azidosalicylamido]ethyl-1,3'-dithiopropionate, *N*-Hydroxysuccinimidyl-2,3-dibromopropionate, *N*-Succinimidyl[4-iodoacetyl] aminobenzoate, Sulfosuccinimidyl[4-iodoacetyl] aminobenzoate, Succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate, Sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate, Succinimidyl 4-[*p*-maleimidophenyl]butyrate, Sulfosuccinimidyl 4-[*p*-maleimidophenyl]butyrate, 4-Succinimidyloxycarbonyl-methyl-a-[2-pyridyldithio]toluene, Sulfosuccinimidyl 6-[a-methyl-a-(2-pyridyldithio)toluamido]hexanoate, *N*-Succinimidyl-3-[2-

pyridyldithio]propionate, Succinimidyl 6-[3-[2-pyridyldithio]propionamido]hexanoate, Sulfosuccinimidyl 6-[3-[2-pyridyldithio]propionamido]hexanoate, Sulfosuccinimidyl 7-azido-4-methylcoumarin-3-acetate, and Sulfosuccinimidyl 4-[*p*-azidophenyl]butyrate

It should be understood, that numerous groups can be used to couple the bioactive agent to the nanoErythrosome. Such groups comprise but are not limited to NH_2, COOH, SH and OH groups, which are found in abundance in the constituents of the nanoErythrosomes.

It will be understood that the coupling agent should not detrimentally interfere with the activity of the bioactive agent and should not render the complex toxic to the host.

Since a multitude of bioactive agents can be conjugated to or entrapped within the nanoErythrosomes of the invention, from the specification and appended claims, it is to be understood that the term bioactive agent is designed to include, but is not limited to photosensitive compounds, drugs, antibiotics, antineoplastic agents, anti-inflammatory agents, proteins or parts thereof, enzymes, nucleotide sequences, nucleic acids or parts thereof, oligonucleotides, antisense, genes, vectors, expression vectors, radioactive isotopes, amino acid analogs or nucleoside analogs, as well as other medically or veterinarilly useful agents such as contrast materials (e.g. dyes) and diagnostic materials as well as growth factors, hormones such as corticosteroids or the like. Furthermore, it is to be understood that the term bioactive agent should be taken in a broad sense so as to also include a combination of at least two bioactive agents.

From the specification and appended claims, the term pharmaceutical, should be understood as including veterinary, since the nanoErythrosomes of the present invention are suited for numerous types of treatment, prophylaxy or diagnosis in mammals.

The nanoErythrosomes of the present invention can also serve as a diagnostic tool. Numerous types of bioactive agents could be coupled to the nanoErythrosomes of the invention, for example antibodies, in order to target a specific tissue or cell type. The detection of the target can be assessed according to known methods, including for example the use of a label, radioactive or not, or a dye entrapped in the nanoErythrosomes. One of numerous examples of the diagnostic use of the nanoErythrosomes of the invention is to target a tumoral antigen, through an antibody specific to this antigen, in order to detect, quantify or analyse the presence of metastases.

The choice of the bioactive agent, and whether it is entrapped in the nanoErythrosome or conjugated thereto will depend on the desired application, the purpose of delivery, the route of delivery, the target, and other parameters relating to the use of the nanoErythrosomes.

Depending upon the purpose of delivery, the nanoErythrosomes may be administered by a number of routes: in man and animals these include but are not limited to injection (e.g., intravenous, intraperitoneal, intramuscular, subcutaneous, intraauricular, intramammary, intraurethrally, etc.), topical application (e.g., on afflicted areas), and by absorption through epithelial or mucocutaneous linings (e.g., ocular epithelia, oral mucosa, rectal and vaginal epithelial linings, the respiratory tract linings, nasopharyngeal mucosa, intestinal mucosa, etc.).

The mode of administration of the preparation may determine the sites and cells in the organism to which the compound will be delivered. NanoErythrosomes can be administered alone but will generally be administered in admixture with a pharmaceutical carrier selected with regard to the intended route of administration and standard pharmaceutical practice. Such preparations may be injected parenterally, for example, intraperitoneally, intra-arterially or intravenously. The preparations may also be administered via oral, subcutaneous, intramuscular and, of course, intraorgan routes. For parenteral administration, they can be used, for example, in the form of a sterile aqueous solution which may contain other solutes, for example, enough salts or glucose to make the solution isotonic. Other uses, depending upon the particular properties of the preparation, may be envisioned by those skilled in the art. Delivery of the nanoErythrosome formulation by way of an aerosol is also contemplated as a method of administration.

For administration to mammals including humans in the curative treatment of disease states, the prescribing medical professional will ultimately determine the appropriate dosage for a given subject, and this can be expected to vary according to the agent, weight, and response of the animal as well as the nature and severity of the disease. The same principle can be applied for a diagnostic use of the nanoErythrosomes. The dosage of the bioactive agent in a nanoErythrosome formulation can, according to the present invention, be lower than that employed for the free bioactive agent. In some cases, however, it may be necessary to administer equal or higher doses. It is also contemplated that periodic treatments or different cycles of treatment might be beneficial.

The route of delivery of nanoErythrosomes can also affect their distribution in the body. Passive delivery of nanoErythrosomes involves the use of various routes of administration, e.g., intravenous, subcutaneous and topical. Each route produces differences in localization of the nanoErythrosomes. Targeting of the nanoErythrosomes and bioactive agent to selected target areas is also contemplated.

Nucleic acid sequences of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones including phosphorothioates, dithionates, alkyl phosphonates and nucleotides and the like. Modified sugar-phosphate backbones are generally taught by Miller, 1988, Ann. Reports Med. Chem. 23:295 and Moran et al., 1987, Nucleic acid molecule. Acids Res., 14:5019. In certain embodiments, the nucleic acid sequence can be radioactively labeled so as to specifically permit radiotherapy.

As used herein, the term "gene" is well known in the art and relates to a nucleic acid sequence defining a single protein or polypeptide. A "structural gene" defines a DNA sequence which is transcribed into RNA and translated into a protein having a specific amino acid sequence thereby giving rise the a specific polypeptide or protein. It will readily recognized by the person of ordinary skill, that the nucleic acid sequence of the present invention can be incorporated into anyone of numerous established kit formats which are well known in the art.

The term "vector" is commonly known in the art and defines a plasmid DNA, phage DNA, viral DNA and the like, which can serve as a DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the art.

The term "expression" defines the process by which a structural gene is transcribed into mRNA (transcription), the mRNA is then being translated (translation) into one polypeptide (or protein) or more.

The terminology "expression vector" defines a vector or vehicle as described above but designed to enable the expression of an inserted sequence following transformation into a host. The cloned gene (inserted sequence) is usually placed under the control of control element sequences such as promoter sequences. The placing of a cloned gene under such control sequences is often referred to as being operably linked to control elements or sequences.

Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

In another embodiment, the present invention relates to a kit comprising a nanoErythrosome of the present invention comprising at least one container means containing a nanoErythrosome composition in accordance with the present invention. In one preferred embodiment, the kit comprises other containers comprising one or more of the following reagents, wash reagents, detection reagents, and the like.

As used herein, the term "antibody" includes monoclonal and polyclonal antibodies as well as fragments thereof, single chain antibodies, antibody fragments which contain the idiotype of a specific molecule and antibody fragments. Thus, the term antibody as used herein

also includes $F(AB')_2$ fragment, the F(ab') fragments, F(ab) fragment and Fv fragments. In general, techniques for preparing and modifying antibodies are well known in the art (Harlow et al., 1988, Antibody - A Laboratory Manual, CSH Laboratories; Campbell, 1984, Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publisher, Amsterdam, The Netherlands).

From the present invention, it will be clear that, the nanoErythrosome compositions have broad therapeutic applications. One non-limiting example of such application includes gene therapy by which a nucleic acid segment can be transferred to a patient. In one embodiment, a nanoErythrosome composition of the present invention permits a specific targeting of an organ, tissue or cell by way of a specific ligand directed against this organ, tissue or cell.

It will be clear that the nanoErythrosome compositions of the present invention can deliver agonists, and antagonists of gene products.

The delivery of antisense molecules, using the nanoErythrosome compositions of the present invention is also contemplated. The specific design of the antisense and modification thereof to increase its stability, are well known in the art.

Suitable pharmaceutical carriers, excipients, and therapeutic or diagnostic compositions in general are described in Remington's Pharmaceutical Sciences, 1980, 16th, Ed., a standard reference in this particular field.

It will be clear the person of ordinary skill, that a wide range of cytotoxic agents and drugs can be used in accordance with the

present invention. Non-limiting examples of cytotoxic agents include toxins such as diphtheria toxin. Further, as mentioned earlier radionuclides can also be coupled to the nanoErythrosome compositions of the present invention to exert the cytotoxic effect by local irradiation of cells and hence to radiotherapy. Radionuclides which can be used in radiotherapy are well known in the art and can be readily adapted to a particular clinical or diagnostic situation.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

Figure 1 shows a summary of the methodologies used to produce nanoErythrosomes to be used in diagnostic and/or therapeutic applications;

Figure 2 shows different PEG molecules which have been produced;

Figure 3 shows a direct agglutination test demonstrating the significant reduction in immunogenic potential of nEryt-PEG compositions; and

Figure 4 shows phase contrast microscopy a and C and fluorescence microscopy B-D of T24 (AB) and EFFRON cells (CD).

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Figure 1 shows a summary of the methodologies used to produce nanoErythrosomes that are involved in diagnostic or therapeutic applications.

The technology of the production of the nanoErythrosomes has been taken a step further by exploring administration thereof through parenteral or anteral routes. For the parenteral route, nanoErythrosomes were used successfully to show that they could identify cancer cells of the bladder. In brief, nanoErythrosomes were concentrated by centrifugation and mixed in a concentrated solution of dextran-fluorocsein isothyocyanate (Dextran-FITC; MW 4000 to 2 X 10^6). After one round of freeze-thawing in liquid nitrogen, nanoErythrosomes which had encapsulated the dextran-FITC were purified either by filtration through size-exclusion, gel using Sephadex or Sepharose (i.e. G-25 or PD10), or alternatively by four cycles of wash-centrifugation. NanoErythrosome membranes were modified by a first reaction with SPDP. The chosen antibody, in this case monoclonal antibody 48-127, which recognizes the cancer antigen TROP-2, an antigen present on a large number of bladder cancer cells, was first modified with SPDP followed by treatment with a reducing agent such as DTT (dithiothreitol) liberate the free SH group. Finally, the modified nanoErythrosomes from above and the monoclonal antibody 48-127, also modified, were mixed together in a physiological buffer in order to favour the formation of a composition termed the nanoErythrosome - Dextran - FITC - 48127. Biological assays using this complex clearly demonstrated that the cells carrying the TROP-2 antigen recognized by 48-127 were

very selectively decorated by the complex while non-TROP-2 carrying cells were not.

Similar types of experiments but replacing Dextran by phototherapeutic agent such as phthalocyanine octacarboxylic, permitted to show in a first stage, that the phthalocyanine was easily captured by the nanoErythrosome using the thermal shock treatment described herein. The second phase, comprising the coupling of the 48-127 antibody to the nanoErythrosome - phthalocyanine has been carried out. Following this coupling, the evaluation of the labelling of the cells and ultimately, pertinent studies to exploit the phototherapeutic properties of phthalocyanine in view of cancer treatment of the bladder *in situ* will be carried out. It is expected that this second type of complex (nanoErythrosome - phthalocyanine - 48-127) will show a significant inhibitory effect on cancer cells.

In a third set of experiments, an immunotoxin such as *Pseudomonas* toxin is being encapsulated in nanoErythrosomes in order to show that the nanoErythrosome-*Pseudomonas* toxin - 48127 complex can be efficiently used as a cytotoxic delivery system of high specificity. These latter two types of complexes may be shown to be useful for intravesicular treatment of various bladder cancers. Such complexes could overcome the main problem encountered with currently used treatment methodologies which is the very short period of contact between the drugs and the targeted bladder cells (this is caused by the rapid accumulation in the bladder of the urine produced by the kidneys).

Taken together, the present invention shows that nanoErythrosomes can encapsulate, using the method of the present invention, molecules having molecular weights ranging from 1000

(phthalocyanine octacarboxylic) to 2 X 10^6 (Dextran). The NanoErythrosome is thus a very versatile drug delivery system. In addition, dextran and phthalocyanine molecules have been shown to be stably encapsulated by the nanoErythrosomes for periods longer than six months.

With respect to therapeutic anteral applications, nanoErythrosomes could eventually permit a *per os* administration of biologically relevant substances such as drugs, enzymes, proteins (i.e. insulin), immunomodulating compounds, peptides or other substances sensitive to the gastro-intestinal system. The strategy used to obtain such a modified delivery system, consists in encapsulating under the thermal shock treatment method described above, a biologically relevant substance inside the nanoErythrosomes. In a second step, derivatives of the Polyethyleneglycol (PEG-cyanuryl chloride or the like) is conjugated to the nanoErythrosome. Such a Polyethyleneglycol derivative is known to abrogate antigenicity and to increase the biological half life of proteins and enzymes. Such a system could thus permit the use of non-autologous and non-homologous nanoErythrosomes.

It should be noted that the invention is not limited to PEG-cyanuryl chloride since numerous other PEG derivatives can be used. Such derivatives are well known to a person of ordinary skill. Non-limiting examples of such PEG derivatives comprise PEG derivatives having activated esters. It follows, that a person of ordinary skill could adapt the teachings of the present invention to other types of PEG derivatives.

In a preferred embodiment, the nanoErythrosomes are conjugated to PEG-5000 - cyanuryl chloride. Although PEG derivatives

between about 350 and 10,000 molecular weight can be used in accordance with the present invention, it should be understood that it is preferable to use a PEG derivative having a molecular weight between about 1,000 and 7,000 and more preferably between about 2,000 and 10,000. The stability of PEG-modified nanoErythrosomes is currently tested using Dextran-FITC encapsulated nanoErythrosomes. Experiments are under way to verify the encapsulation and stability of insulin-I125 and evaluate the biopharmaceutical parameters of such nanoErythrosomes in animals following different types of administration (iv, ip and *per os*). In addition, the capacity of nanoErythrosome - PEG containing insulin or hormone such as LHRH to induce a pharmacological response when administered *per os* also will be tested.

It is expected that all these experiments will demonstrate the versatility and utility of nanoErythrosomes. The demonstration that a peptidic substance, and hence, a substance sensitive to gastric juice and the like, maintains its biological activity when protected by the nanoErythrosome could have a major impact on the *per os* administration of sensitive substances in general.

The present invention is illustrated in further detail by the following non-limiting examples.

EXAMPLE 1

Removal of plasma and buffy coat (white blood cells)

Blood (2 X 30 ml) from mammal donors (echine, bovine, porcine, human, etc) is centrifuged at 500 X g (1500 rpm) for 10 min at 4°C. Plasma and buffy coat are removed by aspiration. The volume removed is replaced by the same volume of Phosphate Buffer Saline (PBS) sodium phosphate 5 mM, sodium chloride 150mM) at pH 7.4 and at 4°C. The mixture is gently homogeneized by several inversion of the tubes. Blood is then recentrifuged again at 500 X g for 10 min at 4°C. The sequence described herein is repeated 3 times.

Each one of the following protocols is conducted under sterile conditions.

EXAMPLE 2

Preparation of erythrocytes ghosts Hypotonic shock

Method 2.1

Five ml of blood treated as described in Example 1 are added to 35 ml (in 50 ml Nalgene™ tubes) of hypotonic buffer (sodium phosphate 5mM) at pH 7.4. The erythrocytes suspension is centrifuged at 25,000 X g for 20 min at 4°C.

The supernatant is aspirated and discarded. The volume of hypotonic buffer removed is replaced by the same volume of fresh buffer and the suspension is centrifuged again. The procedure is repeated 3 times (until the supernatant is slightly colored. The pellet is suspended in 5 ml of PBS buffer at pH 7.4 at 4°C. White ghost suspensions can be pooled, concentrated by centrifugation (20,000 X g) for 20 min, and kept at 4°C until needed.

Method 2.2

Seventy ml of blood, as treated in Example 1, is chromatographied on a Sepharose CL6B (CL4B also) or Sephacryl S400 column (for ex.: 10 cm X 30 cm) using an hypotonic phosphate or carbonate buffer (2-5 mM) (Na3PO4, 2 mM; pH adjusted using glacial acetic acid) at pH 10 to 11. White ghosts are collected first (dead volume) and hemoglobin is retained on the column. The pH is brought at 7.4 using acetic acid and the ghosts can be concentrated by centrifugation as described earlier. The chromatography yield to 70 mg of white ghosts depleted of hemoglobin (1 mg/ml of "washed blood").

EXAMPLE 3

Preparation of nanoErythrosomes (nEryt)

Method 3.1

NanoErythrosomes can be prepared as described previously in USP 5,653,999. It is important to point-out that ghosts are extruded through polycarbonate filters having pores of approximately 1µm. The polycarbonate filter provide the advantage of not sticking to nanoErythrosomes. The pore size is important for the preparation of vesicles. Ghosts are extruded from isotonic suspensions and under nitrogen pressure. The suspension is preferably extruded 4 times.

Method 3.2

This procedure significantly differs from that of 3.1 the previous. It provides the advantages of being faster and giving better yields of nanoErythrosomes. Furthermore, it is more readily avenable to large scale production.

An hypotonic suspension (0.2 mg/ml) of white ghosts in hypotonic PB at pH 7.4 is immediately filtered under vacuum through a polycarbonate (nylon or polyethersulfone) filter having pores of approximately 0.45µm, immediately followed by a second filtration through a polycarbonate (nylon or polyethersulfone) filter having pores of approximately 0.22µm. The procedure must be carried-out in an hypotonic buffer (di and/or trivalent cation-free). The non-compliance to this basic requirement will lead to quickly plugged and unusable filters. Using this methodology only a single passage is necessary and only 5 to 10% of the nanoErythrosomes are lost in the process. The nanoErythrosomes obtained according to method 3.1 have a mean diameter of 100 to 200nm. Light scattering experiments using nEryt suspensions prepared according to method 3.2 three populations of nEryt a) 40-50nm; b) 100 nm; and c) a smaller subpopulation of 200nm. The mean diameter of the nEryt suspension prepared in accordance with method 3.2 remain however at about 100-200 nm.

EXAMPLE 4

Concentration of nanoErythrosomes suspension

nEryt suspensions (350 ml at 0.2 mg of protein per ml) are concentrated using an Amicon™ concentrator (model 8400, membrane having a molecular cut-off smaller than 500 000 (for example ZM 500 and YM 100) to a volume of 50 ml under a nitrogen pressure of 10 psi and a very gentle stirring.

EXAMPLE 5

Entrapment (capture) of molecules by nEryt

A general protocol can be used to capture (entrap) bioactive agents such as proteins (i.e insulin), photodynamic agents (i.e porphyrine-like molecules), fluorophores (i.e dextran (4,000 to 2 000 000 mw)-fluorescein isothiocyanate (FITC)), nucleotides (i.e DNA), glycoproteins, polysaccharides (i.e inulin), vitamins, drugs (i.e. bleomycin) and the like.

In eppendorf tubes, suspension of nEryt (1.5 ml, 1 to 2 mg/ml) are centrifugated (microfuge™) at 16,000 X g for 10 min at 4°C. The supernatant is removed and discarded. The protein content of the pellet is determined according to well known methods (i.e. a commercially available kit). One ml of the concentrated solution of the substance to be capture (entrapped) is added to the pellet. The suspension is gently homogeneized by several inversion of the tubes and kept at 4°C room temperature for 10 min. Afterward, the suspension is frozen in liquid nitrogen for 2 min and unfrozen in a water bath at 25°C. It is important to point out that some capture of molecules such as porphyrine-like molecules, DNA can be observed following incubation at room temperature for 30 min. Nevertheless, the heat-shock treatment significantly improves the capture of the bioactive agent. It should be noted that the heat-shock need not be dependent on a -180°C step, as freezing at -70°C or -20°C can also be used. Ice (0°C) is even sufficient for certain bioactive agents, albeit not as efficient as freezing especially at -180°C.

EXAMPLE 6

Purification of nEryt having capture (entrapped) a molecule

Method 6.1

After the capture treatment, the nEryt are freed from the unentrapped molecules by 3 to 5 cycles of dilution with 1 ml of cold (4°C) PBS (isotonic at pH 7.4), centrifugation at 16,000 X g for 8 min, removal of the supernatant and again dilution with PBS.

Method 6.2

After the capture treatment, the nEryt are freed from the unentrapped molecules by using size exclusion chromatography with the proper Sephadex or Sephacryl column according to well known methods allowing the retardation of free molecules and the rapid elution of nEryt. The captured (entrapped) material can evaluated by usual methodologies (adapted to the molecule being entrapped) after solubilization of nEryt using a suitable detergent.

Stability

The capture results in the formation of a stable nanoErythrosome-bioactive agent composition as evidenced by the fact that suspensions of dextran-FITC-nEryt are stable for at least 7 months when kept at 4°C. Furthermore, entrapment of DNA was carried out. Briefly, two hundred nanograms of nEryt suspended in PBS (500 µl) are washed 3 times with the TKN buffer (to eliminate phosphates). DNA (30 to 320 ng) in TKN buffer (10 to 300 µl) is added to the suspension and gently homogeneized. The suspension is incubated at 4°C for 10 min and frozen in liquid nitrogen for 2 min followed by incubation at 25°C for 4 min. The suspension is treated as described previously using TKNM buffer.

The excess of DNA was quickly removed by incubation of the reaction mixture with Dnase (100 µl of 0.1 U/µl Dnase I solution). Mg^{2+}(10 mM) or Mn^{2+}(1 mM) is added to the mixture.

After cleaning with Dnase, nEryt-DNA was amplified using conventional protocols of PCR and confirmed the presence of the vector within the nEryt.

Table 1

Using the technics described herein, these exemples illustrate typical results of capture by nEryth reflecting similar concentrations of small and large molecules as well.

Initial concentration Dextran-4 000 FITC (mg/mL)	**nEryth (mg/mL)**	**captured molecule (µg/mg of nanos)**
2.6	1.3	15.6
2.6	2.6	13.4
2.6	3.9	12.1
DNA (µg/mL)*		
1.3	4	0.003
13	4	0.066
65	4	0.153

*Similar results were obtained for linear (3 kb) or supercoiled DNA.

Thus, Table 1 shows these examples which illustrate typical results of capture by nEryth reflecting similar concentrations of small and large molecules as well.

EXAMPLE 7

Preparation of samples for the Lyophilization of nEryt

A typical preparation consists of adding 1 ml (1 mg of proteins) of a suspension of nEryt (or nEryt having a captured bioactive agent or a PEG coupled nEryt having a captured bioactive agent or a nEryt antibody on its surface) in either hypotonic PB at pH 7.4 or isotonic PBS. Five hundreds microliters of the following solution lyophilization solution(Lyo sln) [(sucrose (26.3% p/v (770 mM), polyvinylpyrrolidone (mw= 10,000 18.1% p/v; (18 mM), EDTA (ethylenediamine tetraacetic acid trisodium salt; 1 mM= Lyo sln) dissolved in either PBS or PB at pH 7.4] are added. If nEryt are in an isotonic solution then Lyo sln is hypotonic an inversely if nEryt are in hypotonic solutions. nEryt samples are stable to 7 cycles of freezing at -20°C (12 h)-thawing at 25°C (16h) and finally heating at 40°C for 18 hours.

Lyophilization

A typical preparation consisting of 1.5 ml of the suspension of nEryt, as previously prepared, in 10 mL vials is frozen for 2 min in liquid nitrogen. The suspension is then lyophilized for 6 to 12 h using a lyophilizator Freezemobile™ 12EL Virtis from The Virtis Compagny. Addition of a buffered sucrose solution (sucrose 25.5% p/v (745 mM) in water or PBS to lyophilizated nEryt led, after incubation of the suspension at 37°C for 1 h, to nEryt maintaining their function of encapsulating molecules. nEryt having already encapsulated molecules such as dextran-FITC are still functional and very little coalescence is observable in microscopy.

EXAMPLE 8

Conjugation of polyethylenegycols and monoclonal antibodies to nanoErythrosomes

PEG having the following structures were prepared in the laboratory according to known methods:

Conjugation of methoxy PEG-S-succinimidyl succinic ester (mw=2000) (MPEG-S suc-suc-2000) to nEryt. To a suspension of 1.5 mg of nEryt in a final volume of 750 µl of PBS at pH 7.4 are added MPEG-S suc-suc-2000 to obtain a final concentration of 1.83 nM (2.7 mg) The suspension maintained at room temperature for 1 h and gently homogeneized by constant inversion of the suspension. The mixture is purified by size exclusion chromatography over a Sepharose CL6B column. Evaluation of the yield of conjugation has shown 11% of conjugation of MPEG-S suc-suc-2000 to nEryt (LJ. Karr, DL. Donnelly, A. Kozlowski and J. Milton-Harris, 1994, Methods in Enzymology, 228:377).

Conjugation of methoxy PEG-S-succinimidyl succinic ester (mw=2000) (MPEG-S suc-suc-5000) to nEryt. To a suspension of 0.5 mg of nEryt in a final volume of y500 µl of PBS at pH 7.4 are added MPEG-S suc-suc-5000 to obtain a final concentration of 5.79 nM (14.47 mg). The suspension is maintained at room temperature for 1 h and gently homogeneized by constant inversion of the suspension. The mixture is purified by size exclusion chromatography over a Sepharose CL6B column. Evaluation of the yield of conjugation has shown 14% of conjugation of MPEG-S suc-suc-5000 to nEryt (LJ. Karr, DL. Donnelly, A. Kozlowski and J. Milton-Harris, 1994, Methods in Enzymology, 228:377).

Conjugation of methoxy PEG-O-CH_2COOS succinimidyl propionic ester (mw=5000) (MPEG-O-SPAsuc-5000) to nEryt. To a suspension of 0.5 mg

of human nEryt in a final volume of 500 µl of PBS at pH 7.4 are added MPEG-O-SPA-5000 to obtain a final concentration of 0.03 M. The suspension maintained at room temperature for 1 h and gently homogenized by constant inversion of the suspension. The mixture is purified by size exclusion chromatography over a Sepharose CL6B column. Evaluation of the yield of conjugation using fluorescamine showed 30% of conjugation to nEryt (LJ. Karr, DL Donnelly. A. Kozlowski and J. Milton-Harris, 1994,Methods in Enzymology, 228:377).

EXAMPLE 9

Immunogenicity of PEG-coupled nEryt

To monitor the immunogenicity of nEryt preparations coupled to MPEG-S suc-suc-5000 of Example 8, the antibody responses induced by equivalent amounts of the parent horse red blood cells (HRBC), non-PEG conjugated nEryt (nEryt) and PEG conjugated nEryt (nEryt-PEG) were analysed.

Four groups having 3 mice each were immunized intraperitoneally with either 2x10 u.c HRBC, nEryt (conjugated to PEG or not) to a dose of 250mg (of protein), or 200ml of phosphate buffered saline (PBS). One week after the 3rd immunization serum was harvested from each mouse and tested for the presence of antibodies against the parent HRBC in both direct and indirect agglutination tests.

Each serum sample was titrated in PBS and 25ml of each dilution was added to 25ml of a 10% solution of HRBC in individual wells of a 96-well plate (round bottom). These were incubated at room temperature for 15-25 minutes and agglutination was scored using an inverted microscope.

As shown in figure 3, mice immunized with parent HRBC showed 50% maximal agglutination at a serum dilution of approximately 1:300. Mice immunized with nEryt showed a 50% maximal agglutination at a serum dilution of 1:100 and mice immunized with nEryt-PEG showed a 50% maximal agglutination at a serum dilution of approximately 1:10. Thus, there results show that maximal agglutination with nEryt-PEG is indistinguishable from that with non-immune control mice (reflecting the presence of natural antibodies against blood group antigens).

The indirect agglutination test effectively measures the presence of IgM antibodies. In order to measure IgG antibodies, anti-mouse immunoglobulin was added to the HRBC used in the direct test described above after washing the HRBC in 200ml of PBS 3 times with aspiration of the supernatant between each wash. The anti-mouse immunoglobulin was used at a concentration of 1:100. After incubating at room temperature for 15 minutes agglutination was again scored using an inverted microscope. The results from this analysis were that agglutination could now be detected in dilution of 1:100 of nEryt and in dilution 1:10 in the nEryt-PEG and control sera. Therefore, the nEryt-PEG were non-immunogenic since the load of agglutination with neyrt-PEG was comparable to that of the control sera.

EXAMPLE 10

Conjugation of monoclonal antibodies to nEryt using an heterobifunctional PEG

Conjugation of iminothiolane to nEryt

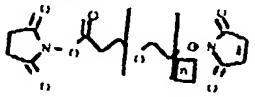
First, a fresh solution of iminoithiolane (IT) is prepared (4.7 M) in PBS-EDTA (5mM K2PO4, 150 mM NaCl, 5 mM EDTA at pH

7.4). The solution is kept on ice. Four milligrams of nEryt are suspended in cold PBS-EDTA buffer and IT solution is added to obtain the ratio 100 µmoles of IT per mg of nEryt in a final solution of 627 mM (IT). The mixture is kept at 4°C in the dark with gentle agitation for 90 min. After the incubation, the mixture is purified on Sephadex G-25 (PD-10). Briefly, the chromatography column is equilibrated with 20 ml of cold PBS-EDTA prior to use. The mixture is applied on the column and eluted with cold PBS-EDTA. The first 2.5 ml is discarded (dead volume) and nEryt are collected in the following 2.5 ml fraction. The samples are kept on ice prior to the next reaction. nEryt-IT are unstable and should be prepared extemporaneously.

EXAMPLE 11

Conjugation of heterobifunctional PEG to monoclonal antibody 48-127

First, a fresh solution of PEG 2000 NHS-MAL



,mw =2000; 3.2 mM)

is prepared. Second, a solution of 48-127 (0.8 mg/ml) in PBS containing 4.7 mM EDTA is prepared. The 200 nmoles of PEG 2000 NHS-MAL are added to 0.4 mg of 48-127 (the final concentration of PEG is 0.4 mM). Monoclonal antibody 48-127 recognizes the gp54 cancer marker at an epitope which differs from that the T16. The solution is incubated for 30 min at 30°C with agitation. The mixture is separated on a PD-10 column previously equilibrated using 5 ml of PBS containing 0.5% of bovine serum albumin and washed with 20 of cold PBS-EDTA buffer. The mixture is applied to the column and eluted with cold PBS-EDTA. The first 2.5 ml is discarded (dead volume) and nEryt are collected in the following 2.5 ml fraction. The samples are kept on ice until the next reaction.

EXAMPLE 12

Conjugation of modified 48-127 to nEryt-IT derivative

To a suspension of nEryt-IT (960 µg) in cold PBS-EDTA buffer (600µl) is added 160 µg (in 1 ml PBS-EDTA) of 48-127 modified with PEG. The mixture is incubated for 18h at 4°C under gentle agitation. The mixture was separated over a Sepharose CL6B column using cold PBS as eluent.

EXAMPLE 13

Conjugation of monoclonal antibodies to nEryt using SMCC as a linking arm

Conjugation of iminothiolane to nEryt:

First, a fresh solution of iminithiolane (IT) is prepared (2 M) in PBS-EDTA. The solution is kept on ice. nEryt are suspended in cold PBS-EDTA buffer and IT solution is added to obtain the ratio 100 µmoles of IT per mg of nEryt (3 mg nEryt in 320 µl of PBS-EDTA, 151 µl of IT in 677 µl of PBS-EDTA). The mixture is kept at 4°C in the dark with gentle agitation for 90 min. After the incubation, the mixture is purified on Sephadex G-25 (PD-10). Briefly, the chromatography column is equilibrated with 20 ml of cold PBS-EDTA prior to use. The mixture is applied on the column and eluted with cold PBS-EDTA. The first 2.5 ml is discarded (dead volume) and nEryt are collected in the following 2.5 ml fraction. The samples are kept on ice prior to the next reaction. nEryt-IT are unstable and should be prepared extemporaneously.

Conjugation of SMCC to monoclonal antibody 48-127.

First, prepare a fresh solution of succinimidyl 4-(n-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, 3.25 mg/ml

DMSO (10mM)) in DMSO. The solution is kept at room temperature. A solution of 48-127 (1 mg) in PBS (700 µl) containing 4.7 mM EDTA is prepared. One hundred and twenty five nanomoles of SMCC are added, the volume of the reaction is 712 µl. The solution is incubated for 30 min at 30°C with agitation. The mixture is separated on a PD-10 column previously equilibrated using 5 ml of PBS containing 0.5% of bovine serum albumin and washed with 20 ml of cold PBS-EDTA buffer. The mixture is applied on the column and eluted with cold PBS-EDTA. The first 2.5 ml is discarded (dead volume) and nEryt are collected in the following 2.5 ml fraction. The samples are kept on ice prior to the next reaction.

Conjugation of modified 48-127 to nEryt-IT derivative. To a suspension of nEryt-IT (3 mg) in cold PBS-EDTA buffer (2.5 ml) is added 0.66 mg of 48-127 modified with SMCC in PBS-EDTA buffer (1.6 ml). The mixture is incubated for 24 h at 4°C under gentle agitation. The mixture was purified over a Sepharose CL6B column using cold PBS as eluent.

EXAMPLE 14

Immunofluorescence using the nEryt-SMCC-48127 composition

Cells carrying the gp54 antigen against which monoclonal antibody 48-127 reacts, or EFFRON cells not expressing gp54 (negative control) were prepared for immunofluorescence according to well known methods.

The nEryt-SMCC-48127 composition was treated as previously described (Example 6). The resulting nEryt (FITC)-SMCC-48127 composition (15 mg/ml) was incubated with T24 cells and EFFRON cells according to known methods.

Briefly, T24 cells and EFFRON cells were used after 120 and 50 passages, respectively. Cells were grown to confluence for 5 days and lifted off the culture dish using PBS containing 2% EDTA and 2% Dextrose. 5 X 10^5 cells were transferred to a sterile slide of 22 cm^2 present at the bottom of a dish of 6 walls. The culture medium used was MEM containing 7.5% phyto serum and antibiotics. The cells were grown for 4 days at 37°C in the presence of 5% CO_2. This following blocking of non-specific sites using MEM containing 7.5% CAV serum and 5% clot serum, the nEryt-Dextran-FITC-48127 composition was fixed on the cells (15 µg/m). The cells and nanoErythrosome composition was incubated for 1h at 37°C in the presence of 5% CO_2. The slides were washed with MEM containing CAV serum (3 ml) and PBS (2 X 3 ml). The slides were mounted on microscopic slid and rinced with 2 ml of PBS.

Figure 4 shows the immunofluorescence results. Briefly, Fig. 4A and 4C show the phase contrast microscopy (400 X) of the T24 and EFFRON cells, respectively. Fig. 4B and 2D show the same microscopy field as 4A and 4C under the fluorescent microscope. As clearly demonstrated, the nEryt-Dextran-FITC-48127 (SMCC) composition has preserved the integrity of the nEryt has seen by the rounded shape of the vesicles as well as the biological activity of the 48-127 antibody which specifically recognizes the gp54 Ag-expressing cells (T24). This results therefore demonstrate the targetability of nEryt to specific cells. It should noted that similar results were obtained using nEryt-SMCC-48127 which we then treated in accordinace with the capture method of Example 6.

EXAMPLE 15

Conjugation of monoclonal antibodies to nEryt using SPDP as a linking arm

Conjugation of N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) to nEryt. First, prepare a fresh solution of N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP, 20 mM (1 mg SPDP) in methanol (160 µl). Three milligrams of nEryt are suspended in 500 µl of PBS at pH 7.4 and 150 µl (1 µmole) of SPDP are added. The suspension is incubated for 30 min at 30°C with agitation. The mixture is purified on a PD-10 column previously equilibrated using 20 ml of cold PBS at pH 7.4. The mixture is applied on the column and eluted with cold PBS. The first 2.5 ml is discarded (dead volume) and nEryt are collected in the following 2.5 ml fraction. The samples are kept on ice prior to the next reaction.

Conjugation of SPDP to monoclonal antibody 48-127. First, prepare a fresh solution of N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP 20 mM (1 mg SPDP) in methanol (160 µl). The solution is kept at room temperature. A solution of 48-127 (2.5 mg) in PBS (425 µl) is prepared. Three hundred and twelve nmoles of SPDP (17 µl) are added to the 48-127 solution. The solution is incubated for 30 min at 30°C with agitation. The mixture is separated on a PD-10 column previously equilibrated with 20 ml of cold acetate buffer (sodium acetate 100 mM; NaCl 145 mM) at pH 4.5. The mixture is applied on the column and eluted with cold acetate buffer. The first 2.5 ml is discarded (dead volume) and nEryt are collected in the following 2.5 ml fraction.

The samples are kept on ice prior to the next reaction. The dithiopyridyl moiety of the molecule is reductively cleaved by incubation for 20 min at room temperature of the suspension (3.6 ml) of 48-127-SPDP collected from the column with 190 µl of a DTT solution (1M solution of dithiotreitol

in acetate buffer at pH 4.5). The mixture is purified over a PD-10 column equilibrated with a cold buffered solution containing 10 mM Hepes and 145 mM NaCl at pH 7.4.

Conjugation of modified 48-127 to nEryt-IT derivative. To a suspension of nEryt-SPDP (3 mg) in cold PBS at pH 7.4 (2.5 ml) is added 1mg of 48-127 antibody modified with SPDP. The mixture is incubated for 18 h at 4°C under gentle agitation. The mixture was separated over a Sepharose CL6B column using cold PBS at pH 7.4 as eluent.

These reactions have also been performed using nEryt having "capture" dextran-FITC (4000 to 2,000,000 mw); the modified nEryt compositions were shown to be still active and stable.

Conclusion

The present invention thus teaches a simple, efficient, quantitative method for the preparation of nanoErythrosomes. NanoErythrosomes thereby produced, in accordance with the teachings of USP 5,653,999 can entrap or capture various bioactive agents. A new method of capture of bioactive agents by nanoErythrosomes is also disclosed. The nanoErythrosomes of the present invention, entrapping or being linked to a bioactive agents can be further modified by pegilation (substitution of groups on a membrane of a nanoErythrosome by PEG - derivatives). Several PEG derivatives were prepared and conjugated to nanoErythrosomes. Such nanoErythrosome-PEG substituted compositions were shown to abrogate immunogenicity of the nanoErythrosome. Further, heterobifunctional PEG bearing a monoclonal antibody were conjugated to nanoErythrosomes having an entrapped dextran-fluorochrome conjugate, and in vitro results showed that this

PEG bearing monoclonal antibody-nanoErythrosome compositions were specifically decorating cells expressing the antigen recognized by the antibody.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

WHAT IS CLAIMED IS:

1. A nanoerythrosome (nEryt)-polyethyleneglycol (PEG) conjugate of the formula:

$$Z\text{-}(YQ)\text{-}X\text{-}(CH_2\text{-}CH_2\text{-}O)_m\text{-}(CH2)_n\text{-}W\text{-}R$$

wherein a) Z is a member selected from the group consisting of COOH, HO (aldehyde), H, OH (hydroxyl), NH_2, and SH, when YQ= CH=CH or -C(R")=CH_2, or H, N_3, OH, CH_3, -NH-NH_2 an anhydride, a mixed anhydride or an activated ester , when Y= -C=(O)-;

b) YQ is a member selected from the group consisting of: CH=CH, -C(R")=CH_2 , (where R'= lower alkyl of 1 to 5 carbon atoms), cyanuric chloride, cyanogen halides (Br or Cl), and OH-activating agents comprising mesyl, and tosyl groups;

c) O(CH_2-CH_2O)m is a polyethyleneglycol derivative where m= 2 to 500;

d) W is a member selected from the group consisting of: O, N, S, -C(=O).

2. The nanoerythrosome (nEryt)-polyethyleneglycol (PEG) conjugate of claim 1, wherein Y is a member selected from the group of lower alkyl [$(CH_2)_n$; n = 1-7], and Q= -C=(O), N, S.

3. The nanoerythrosome (nEryt)-polyethyleneglycol (PEG) conjugate of claim 1 or 2, wherein W= -O-, and R is a member selected from the group consisting of: H, lower alkyl or cycloalkyl of 1 to

7 carbon atoms, -C(=O)-R, where R is a polyamine derivative such as spermine, spermidine or putresceine.

4. The nanoerythrosome (nEryt)-polyethyleneglycol (PEG) conjugate of claim 1 or 2, wherein W= -N-, and R is a member selected from the group consisting of: H, lower alkyl or cycloalkyl of 1 to 7 carbon atoms, -C(=O)- -C(=O)-R, where R is a polyamine deriving from spermine, spermidine or putresceine or a lower alkyl chain of 1 to 6 carbon atoms bearing one or two COOH, SO_3H or PO_4 groups.

5. The nanoerythrosome (nEryt)-polyethyleneglycol (PEG) conjugate of claim 1 or 2, wherein W= -S-, and R is a member selected from the group consisting of: H, lower alkyl or cycloalkyl of 1 to 7 carbon atoms, -C(=O)- -C(=O)-R, where R is a polyamine deriving from spermine, spermidine or putresceine.

6. The nanoerythrosome (nEryt)-polyethyleneglycol (PEG) conjugate of claim 1 or 2, wherein W= -C(=O)-, and R is a member selected from the group consisting of: H, lower alkyl or cycloalkyl of 1 to 7 carbon atoms, -O- -C(=O)-R, where R is a polyamine deriving from spermine, spermidine or putresceine.

7. The nanoerythrosome (nEryt)-polyethyleneglycol (PEG) conjugate of claim 1 or 2, wherein WR is a member selected from the group consisting of: COOH, PO_4, SO_3H.

8. The neryt-PEG conjugate of claim1, wherein YQ is cyanuric chloride, and $(CH_2\text{-}CH_2O)_m$ has a molecular weight of about 350 to about 10,000 and $\text{-}(CH_2)_n = CH_3$.

9. The nEryt-PEG conjugate of claim8, wherein YQ is cyanuric chloride, and $(CH_2\text{-}CH_2O)_m$ has a molecular weight of about 1,000 to about 7,000 and $\text{-}(CH_2)_n = CH_3$.

10. The nEryt-PEG conjugate of claim 8, wherein YQ is cyanuric chloride, $(CH_2\text{-}CH_2O)_m$ has a molecular weight of about 2,000 to about 5,000 and $\text{-}(CH_2)_n = CH_3$.

11. The nEryt-PEG conjugate of claim 1, wherein YQZ is selected from the group consisting of $(CH_2)_4\text{-}COOH$, $(CH_2\text{-}CH_2O)_m$, m= 7, $\text{-}(CH_2)_n$, n= 1 (CH_3), $(CH_2)_3\text{-}CHO$, $(CH_2\text{-}CH_2O)_m$,$(CH_2)_3CHO$, $(CH_2\text{-}CH_2O)_m$, $(CH_2\text{-}CH_2O)_m$, and $(CH_2\text{-}CH_2O)_m$.

12. The nEryt-PEG conjugate of claim1, wherein YQ is selected from cyanuryl chloride derivatives, aldehyde, succinimide, succinimide benzimidazole, symetric disulfide and heterobifunctional PEG, and $(CH_2\text{-}CH_2O)_m$ has a molecular weight of about 350 to about 10,000 and $\text{-}(CH_2)_n = CH_3$.

13. A nEryt-ligand composition, wherein said nEryt is coupled to an antibody or portion thereof, and wherein the antibody or

portions thereof targets said nanoErythrosome to an antigen recognized by the antibody or portion thereof.

14. A nanoErythrosome composition having a significantly reduced immunogenic potential, wherein said composition comprises a PEG derivative conjugated to said nanoErythrosome.

15. A method of producing nanoErythrosomes from red blood cells from a mammal, said method comprising size-exclusion chromatography on a column containing an appropriate chromatographic gel, using a hypotonicaqueous buffered solution at pH 8 to 1, without di and/or trivalent cations.

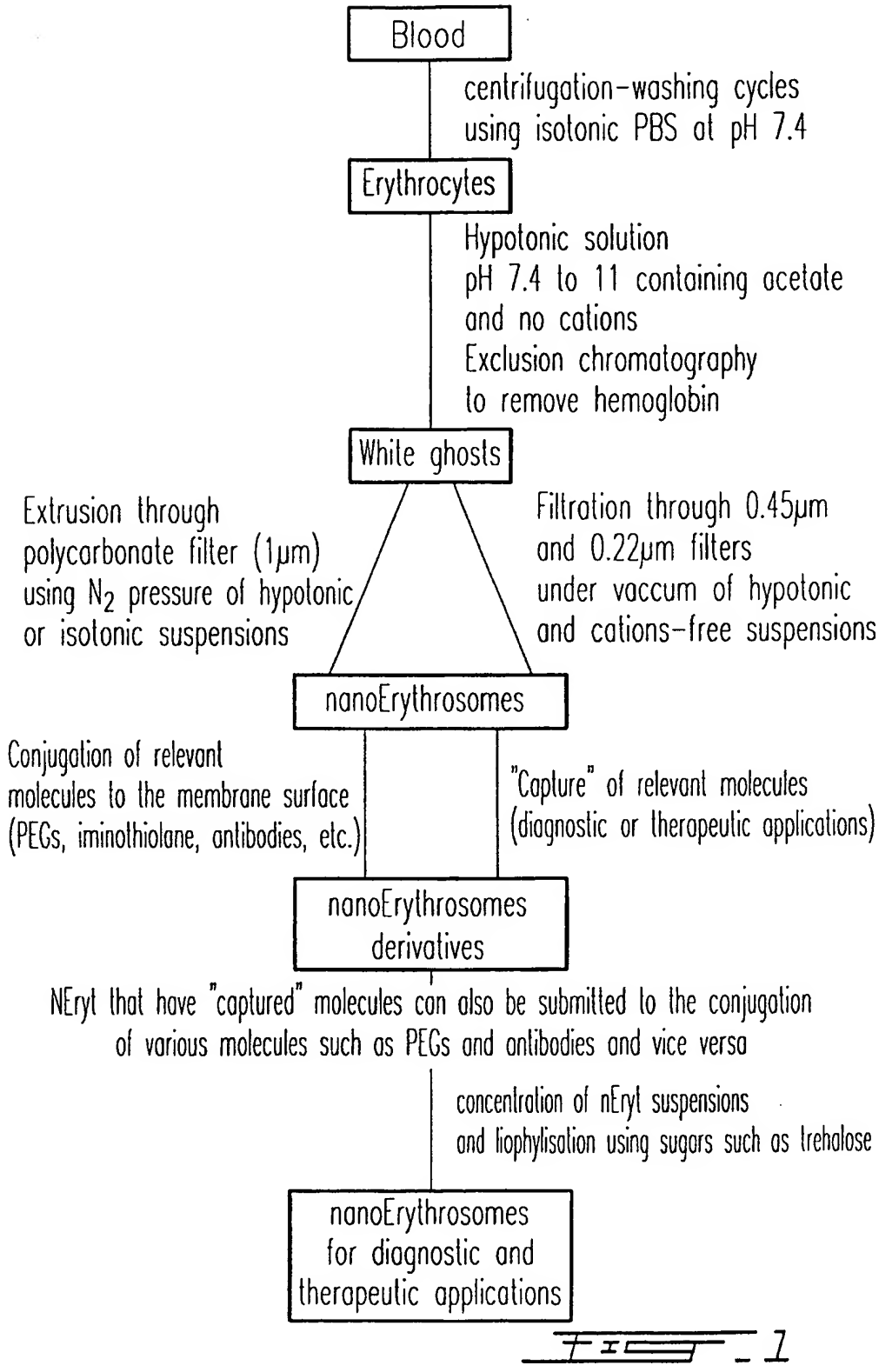

Blood
centrifugation-washing cycles
using isotonic PBS at pH 7.4
Erythrocytes
Hypotonic solution
pH 7.4 to 11 containing acetate
and no cations
Exclusion chromatography
to remove hemoglobin
White ghosts
Extrusion through
polycarbonate filter (1µm)
using N_2 pressure of hypotonic
or isotonic suspensions
Filtration through 0.45µm
and 0.22µm filters
under vaccum of hypotonic
and cations-free suspensions
nanoErythrosomes
Conjugation of relevant
molecules to the membrane surface
(PEGs, iminothiolane, antibodies, etc.)
"Capture" of relevant molecules
(diagnostic or therapeutic applications)
nanoErythrosomes
derivatives
NEryt that have "captured" molecules can also be submitted to the conjugation
of various molecules such as PEGs and antibodies and vice versa
concentration of nEryt suspensions
and liophylisation using sugars such as trehalose
nanoErythrosomes
for diagnostic and
therapeutic applications

Fig - 1

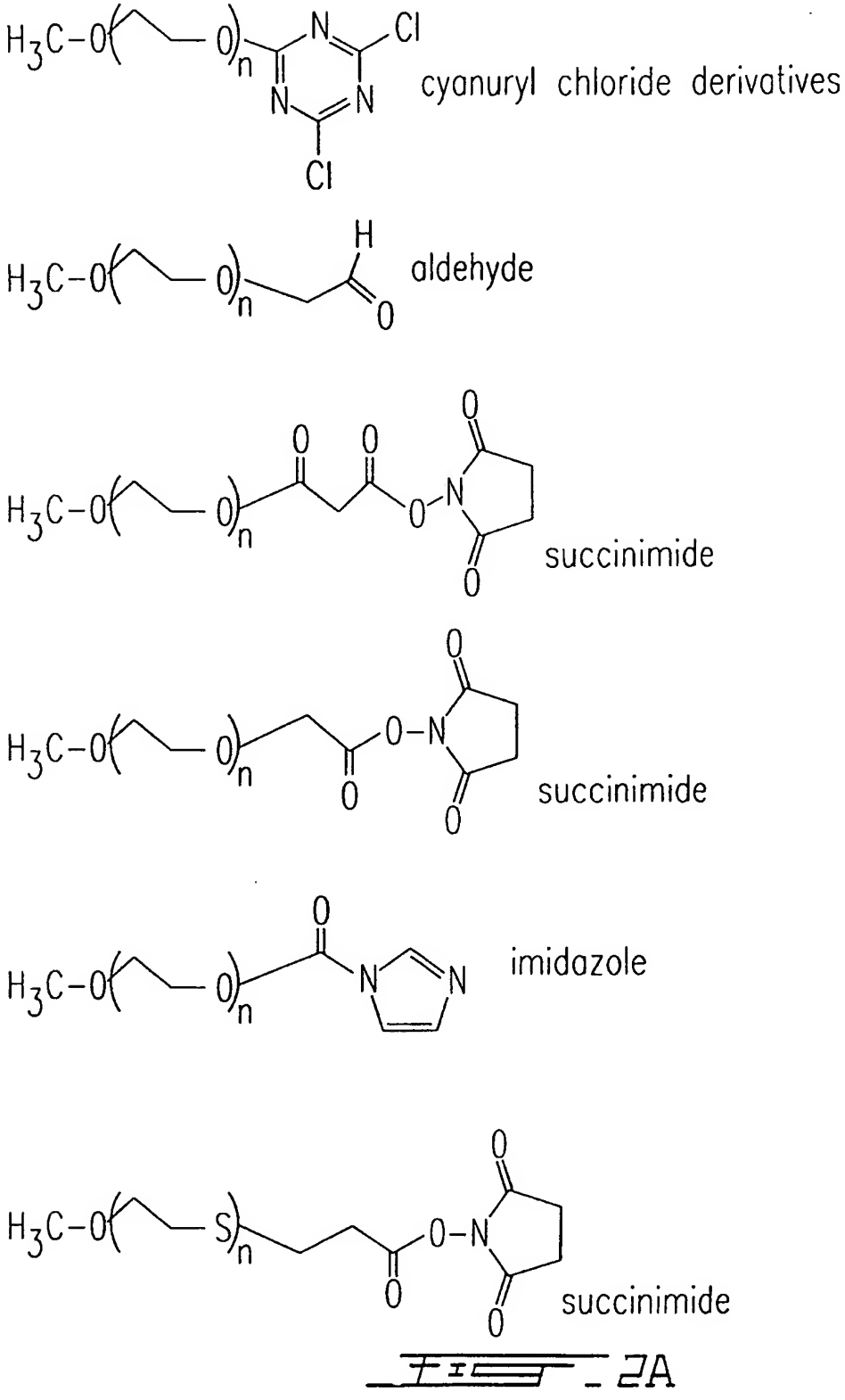
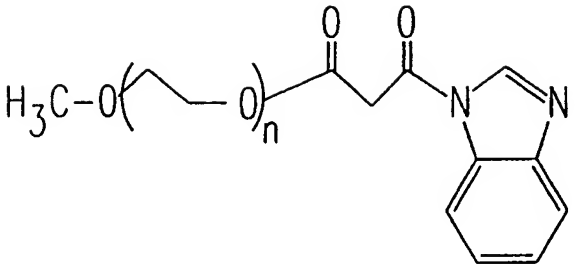
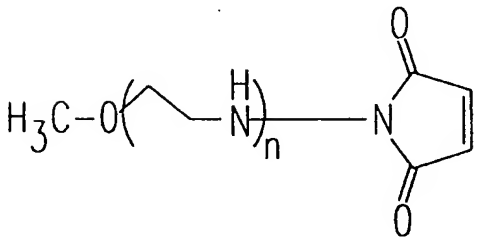

cyanuryl chloride derivatives
aldehyde
succinimide
succinimide
imidazole
succinimide

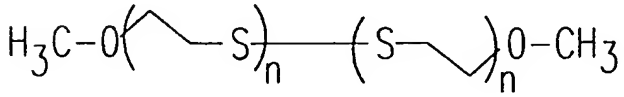
FIG 2A



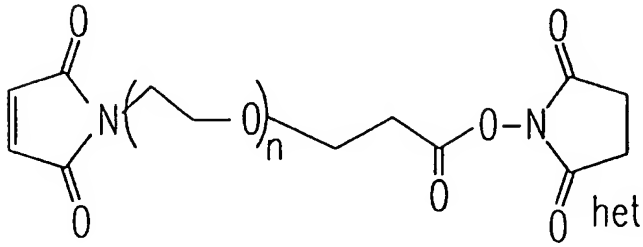
benzimidazole



maleimide



symetric disulfide



heterobifunctional PEG

Fig 2B

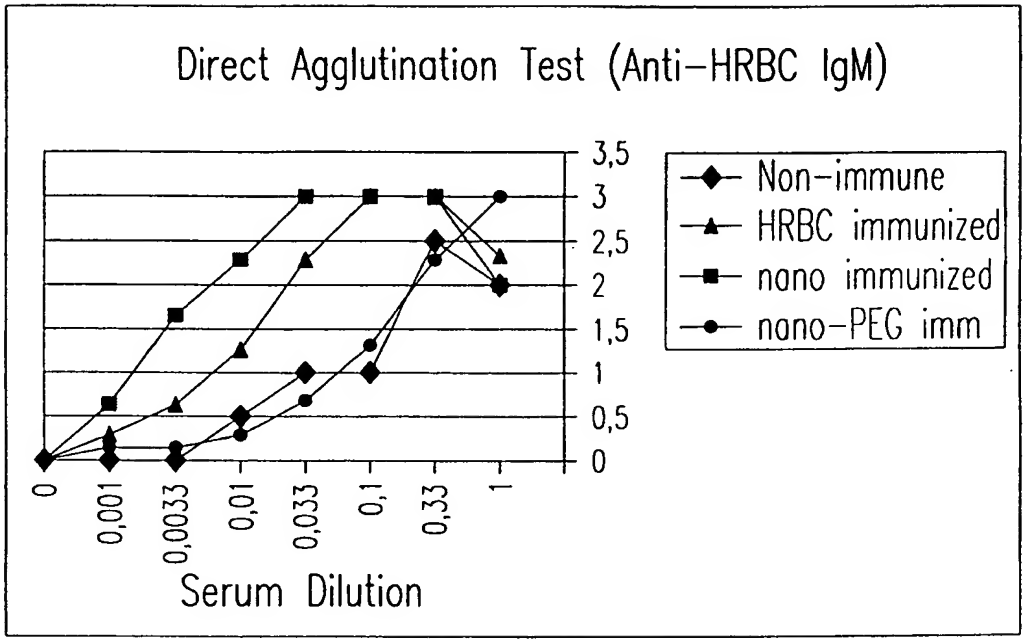

Direct Agglutination Test (Anti-HRBC IgM)
3,5
3
2,5
2
1,5
1
0,5
0
Non-immune
HRBC immunized
nano immunized
nano-PEG imm
0
0,001
0,0033
0,01
0,033
0,1
0,33
1
Serum Dilution

FIG 3

FIG_4A

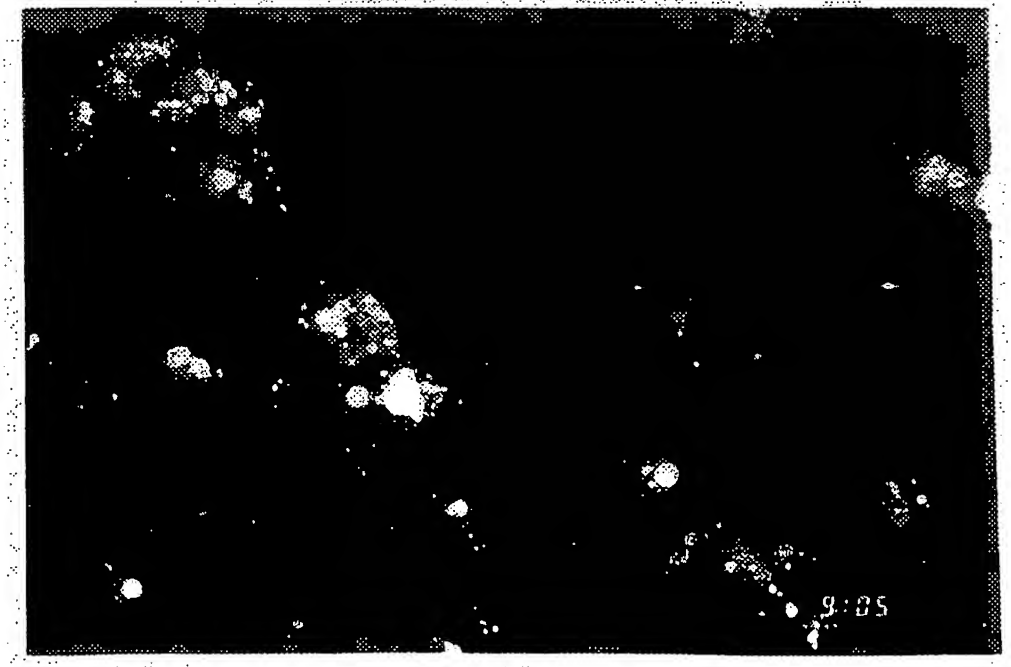


FIG_4B

Fig 4C

Fig 4D